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Conserved Regions as Markers of Different Patterns of Expression and Distribution of the Mucin-Associated Surface Proteins of *Trypanosoma cruzi*

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The MASP gene family is the second most widely represented gene family in the genome of *Trypanosoma cruzi*. One of its main characteristics is that its 5′ and 3′ regions are highly conserved. We assessed the expression of these conserved regions as a marker for *T. cruzi* and also analyzed the expression of the *masp* genes and MASP proteins. In parasite strains CL-Brener (DTUVI lineage) and PAN4 (DTUI lineage), *masp* genes were expressed at different levels both with regard to the two strains and between stages in the parasite's life cycle. We also studied the expression of the family during the intracellular cycle of *T. cruzi*, using antibodies against the conserved MASP signal peptide (SP). Fluorescence intensity showed an increase in expression from 24 h onwards, with a peak in intensity at 72 h postinfection. After 24 and 48 h, the MASP proteins were expressed in 33.33% and 57.14% of the amastigotes, respectively. Our data show that not only the extracellular forms of *T. cruzi* but also the intracellular phases express this type of protein, though to different extents in the various forms of the parasite.

Trypanosoma cruzi is the etiological agent of American trypanosomiasis, or Chagas' disease, which affects some 16 to 18 million people in 21 countries in Central and South America. The disease has spread in recent years to countries such as the United States and Canada, and even to Europe and Australia, as a result of human migration from Latin America (24, 27). The biological cycle of *T. cruzi* involves hematophagous insects of the family Triatominae, in which the parasite multiplies in the epimastigote (E) form within the intestine. The infective stage, the metacyclic trypomastigote (M), develops in the insect's rectum and rectal ampolla, from whence the parasite is expelled during defecation onto the vertebrate host, upon whose blood the insect is feeding. Within the vertebrate host, the parasite goes through two phases: the trypomastigote stage (T), which can be ingested by a new insect vector or can invade any nucleated cell of the host, where it multiplies in the intracellular aflagellate amastigote (A) form, which after multiplying and occupying the cytoplasm of the cell is transformed once again into the T form. Finally, it is released to circulate through the bloodstream and invade new cells, thus completing the parasite's life cycle within the mammal (6).

When the genome of *T. cruzi* was sequenced (12, 13), a number of multigene families were discovered spread extensively throughout the genome, including the genes encoding the MASP family of proteins (12). This is the second largest gene family (containing 1,377 genes), only 14 members of which have been identified to date, by proteomic techniques (2, 3, 14). The simultaneous expression of multiple variables was recently demonstrated after sequencing of a cDNA library of the blood trypomastigote stage, while MASP expression during the amastigote stage had already been described by the same authors (4). The function of these proteins is unknown, although they may be glycosylates; they have a central region that is hypervariable, both in length and in sequence, two highly conserved N- and C-terminal regions, and a glycosylphosphatidylinositol (GPI) binding site. This suggests that although some are secreted by the parasite, most remain bound to the parasite membrane (2, 4). Some of the researchers in our group recently identified the involvement of a protein from this family (MASP52) that is secreted during the process of cell invasion by the parasite (11).

We describe here the conservation of the 3′ and 5′ regions of *masp* genes and also the time course of expression, using the N-terminal conserved region of MASP proteins and genes, which we used to compare phases and strains of *T. cruzi* as well as the intracellular cycle of *T. cruzi*, using quantitative reverse transcription-PCR (qRT-PCR) and immunocytochemical techniques.

MATERIALS AND METHODS

***T. cruzi* culture.** We used the CL-Brener (DTUVI lineage) and PAN4 (DTUI lineage) strains of *T. cruzi*. The infective M and A forms were obtained in modified Grace medium and purified by density gradient centrifugation, respectively, as described previously (9, 11, 18, 23). All forms of the biological cycle of *T. cruzi* used in the assays were at least 95% pure, as tested by microscopic observation of Giemsa staining.

Cell culture. The cells used in the experiments were of the Vero cell line ATCC CCL-81 from the cell culture facilities of the University of Granada (Spain). The cells were cultured at 37°C (pH 7.2) in a humid atmosphere supplemented with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum previously inactivated at 56°C for 30 min (IFCS).

Inoculation of cultures. Semiconfluent Vero cell cultures and a suspension of M forms (5×10^6 forms/ml) were synchronized by treatment with 25 mM thymidine for 9 h as described elsewhere (20). The cells and parasites were then washed with phosphate-buffered saline (PBS), and infection was undertaken for 4 h at 37°C in DMEM (pH 7.2) without ICFS at a parasite/cell ratio of 5:1. Subsequently, the cultures were washed with

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culture medium to remove the forms that had not penetrated, and the infected cells were cultured with DMEM plus 10% IFCS (pH 7.2) at 37°C in order to study the intracellular cycle of the parasite.

Parasites and total DNA and RNA extraction. The stock strains and different stages in the life cycle of *T. cruzi* were obtained as described above. The E stage of the parasite was used for extraction of genomic DNA (gDNA), using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). We used all four stages of the parasite (E, M, T, and A) to extract total RNA, using an RNeasy minikit (Qiagen, Hilden, Germany). The concentration and purity of DNAs and RNAs were measured in a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

qRT-PCR. One microgram of RNA extracted from each stage of *T. cruzi* was used as a mold to synthesize cDNA by employing random primers from an iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Inc.). Expression of the *masp* family was quantified by amplifying a 90-bp fragment from the preserved 5′ terminus, using the primers 5′*masp* F (5′ ATGGCGATGATGATGACTGG 3′) and 5′*masp* R (5′ AACCACACCAA CAGCTCC 3′). As a reference, we used an amplicon of 179 bp of the ribosomal 18S rRNA gene from *T. cruzi* (GenBank accession no. X53917), obtained with the V1 and V2 primers. We quantified the levels of expression by using a Sensimix dT kit (Quantace) according to the maker's instructions, and PCR was carried out in a C-1000 thermocycler connected to a real-time CFX96 module (Bio-Rad Laboratories, Inc.). Relative expression was quantified according to the ΔC_T method, in which the ratio 18S/5′ MASP $= 2^{\text{CT 18S} - \text{CT 5′}masp}$. All assays were carried out in triplicate.

Design and synthesis of peptides and production of polyclonal antibodies. To design the consensus sequence corresponding to the signal peptide (SP) of the MASP proteins, we obtained SPs from 250 MASP sequences from the TriTrypDB (http://tritrypdb.org/tritrypdb/) *T. cruzi* genome database by using the SignalP program (http://www.cbs.dtu.dk/services/SignalP) and then the MEME program (http://meme.sdsc.edu/meme/intro.html). The consensus SP of the MASP proteins (MAMMMTGRVLLVCALCVLW) was synthesized by Genscript.

The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used prior to synthesis to ensure the specificity of the consensus sequence of the SP of the MASP proteins.

Antiserum against the SP was prepared by intraperitoneal injection of BALB/c mice with 50 μg of peptide linked to keyhole limpet hemocyanin (KLH), along with Freund's complete adjuvant, as described elsewhere (25), followed by boosters with Freund's incomplete adjuvant 2 and 4 weeks later. The mice were bled 2 weeks after the last booster. Test bleed sera were checked by indirect enzyme-linked immunosorbent assay (ELISA) in multiwell plates coated with 10 μg/well of the synthetic peptide in 0.1 M bicarbonate coating buffer (pH 8.6). Sera with titers higher than 1:1,600 were pooled and stored at −80°C for later use.

The IgGs from the polyclonal serum against the synthetic peptide and from nonimmunized control mice were purified using a protein A HP spin trap kit (GE Healthcare). The specific IgGs against the signal peptide region were called anti-SP. The protein concentrations were determined by the Bradford method, as described elsewhere (5).

Infection and immunofluorescence assays. To quantify MASP protein expression by immunofluorescence during the infection cycle, the infected cell cultures were fixed with 2% formaldehyde in PBS for 30 min, washed four times in PBS, and permeabilized with PBS plus 0.1% Triton X-100 for 4 min. Fixation was carried out at 2, 24, 48, 72, and 96 h postinfection. Once they were fixed and washed with PBS, the different preparations were incubated for 30 min in blocking buffer (PBS, pH 7.2, 1% bovine serum albumin [BSA]) and treated for 1 h with the anti-SP IgGs at a dilution of 1/100 in blocking buffer at room temperature. An anti-mouse antibody labeled with fluorescein isothiocyanate (FITC; Sigma), diluted in blocking buffer according to the manufacturer's recommendations, was used as a secondary antibody, with which the preparations were also incubated for 1 h at room temperature. For DNA staining, the fixed culture samples were finally treated for 15 min in a 10-μg/ml solution of DAPI (4′,6-diamidino-2-phenylindole). They were subsequently preserved and mounted in mounting medium (Prolong antifade kit; Molecular Probes) and then examined under a Leica DMI6000 confocal laser microscope equipped with a filter system for FITC (maximum wavelength, 490 nm). In some preparations, orthogonal images were processed to pinpoint the location of the fluorescence.

Fluorescence was quantified using ImageJ software (http://rsb.info.nih.gov/ij/). A gating region for the green fluorescence channel was determined, and 100 parasites corresponding to the different assay times were analyzed using the same settings.

Statistical analysis. The Tukey-Kramer test of the software package Graph Pad InStat, version 3.05 (32 bit), was used to study the significance levels for the different hours of infection assayed. P values of <0.05 (*) were taken to be significant, and P values of <0.001 (***) were considered extremely significant. The data in the figures represent means ± standard deviations for measurements made with 100 amastigotes from random samples.

RESULTS

N- and C-terminal conservation of *masp* sequences and *masp* gene expression. We designed primers for the 3′ and 5′ conserved regions of *masp* genes in order to study the extent of conservation of these motifs in the strains of *T. cruzi* examined in our assays (CL-Brener [DTUVI lineage] and PAN4 [DTUI lineage]). We found only one product for each set, of 90 bp (5′) or 71 bp (3′), demonstrating that these regions are the same in these two strains (Fig. 1A). Our search for similar conserved regions in the *masp* family in other kinetoplastids, such as *Trypanosoma rangeli*, *Leishmania major*, and *Blastocrithidia* sp., was unsuccessful (Fig. 1B).

For expression studies, we used the conserved 5′-terminal region, encoding the signal peptide of the MASP proteins. We were able to amplify this region from all stages of the two strains used and found that expression levels varied among both strains and stages. Expression in all stages of the PAN4 strain varied highly significantly ($P < 0.001$) compared to that in the T stage, while the difference between the M and A stages was slightly significant ($P < 0.05$). Compared to the E stage, expression in the T stage was 3.94-fold higher, that in the M stage was 1.32-fold higher, and that in the A stage was 1.18-fold higher (Fig. 2A).

In the CL-Brener strain, there were highly significant differences between the E and the T and A stages, between the M and T stages and the A stage, and between the T and A stages. No significant differences were found between the E and M stages. Compared to the E stage, expression in the M stage was 32.22-fold higher, that in the T stage was 1,795.98-fold higher, and that in the A stage was 6,494.48-fold higher (Fig. 2B).

Therefore, the PAN4 strain was more stable than CL-Brener in its expression profile at the different stages, although a larger quantity of transcripts was produced at the T stage. The CL-Brener strain showed a marked increase in expression in the T stage, with an even greater increase in the A stage, and finally, there was a dramatic decrease in the E and M stages.

Expression of the MASP protein family during the intracellular cycle of *T. cruzi*. An analysis of the SPs corresponding to 250 MASP protein sequences revealed a sequence of 19 amino acids with a high degree of conservation among the members of the family, accounting for all of the SPs of the members of this family. Having located the SP of the MASP proteins by confocal microscopy, we obtained a specific and localized fluorescence in the cytosol of the parasites (Fig. 3). The anti-SP antibody was assayed at 2, 24, 48, 72, and 96 h postinfection. It can be seen in Fig. 4 that

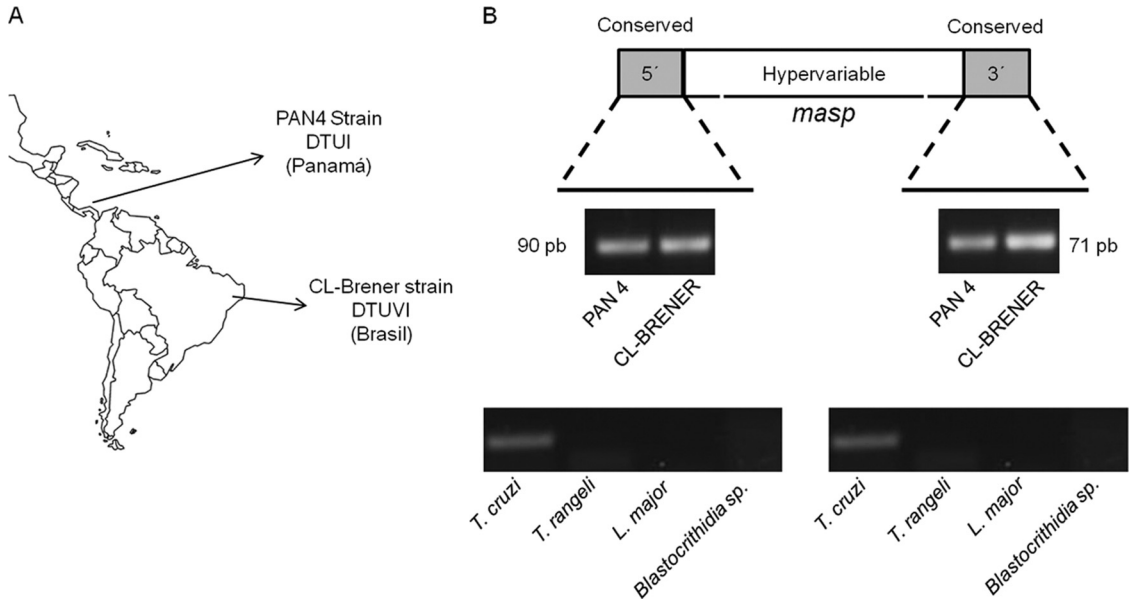

FIG 1 Analysis of the conserved 5′ and 3′ regions and the hypervariable regions of MASPs. (A) Geographic origins and lineages of the strains used in this study. (B) Agarose gels (2%) with amplified products of the preserved 5′ and 3′ regions of the MASPs obtained from the DNAs of the PAN4 and CL-Brener strains of *T. cruzi*, together with those from the other kinetoplastids assayed.

MASP proteins were expressed in greater quantity between 24 and 96 h postinfection, though there were differences in the intensity of the fluorescence signal. The fluorescence levels obtained using the same dilution of antibodies and the same interaction times registered highly significant differences ($P < 0.001$) between the M stage, which had not yet been infected and remained extracellular, and forms that were intracellular or in the process of internalization at 2 h postinfection. This maximum significance value with regard to the forms also held true for all the other times studied during the infection period.

The fluorescence found in the amastigote forms that expressed MASP proteins was also highly significant between 48 and 72 h, as well as between 72 and 96 h, while the differences were less significant ($P < 0.05$) between 24 and 48 h postinfection. An analysis of the overall data confirmed a clear increase in fluorescence from 24 h onwards during the intracellular infection cycle.

At 24 and 48 h postinfection, only 33.33% and 57.14% of the amastigotes of the sample, respectively, expressed MASP proteins (Fig. 3). For the remaining times, i.e., 2, 72, and 96 h (previous and later periods of parasite division), 100% of the parasites were found to express this type of proteins.

DISCUSSION

The MASP gene family accounts for roughly 6% of the *T. cruzi* genome (12). This family does not possess any genes orthologous to those of other sequenced kinetoplastids, which means that they are highly specific to *T. cruzi*. This indicates that some of the biological characteristics unique to this parasite might be attributable to the activity of the *masp* genes in the *T. cruzi* genome. As mentioned above, the functions of some of the encoded proteins may be related to the invasion of the host cell (11) and may also play a part in the mechanisms that allow the protozoan to adapt to the

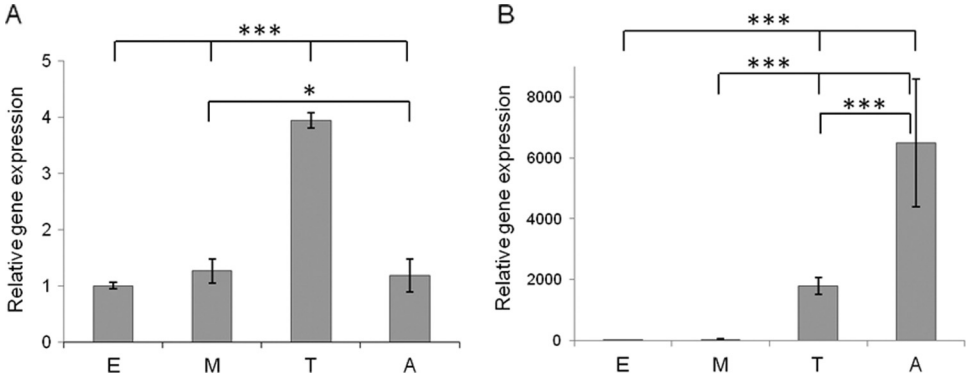

FIG 2 Expression of *masp* genes during different stages of the PAN4 (A) and CL-Brener (B) life cycles. Relative quantities of mRNA of all the transcripts were compared to that of the 18S ribosomal gene in metacyclic trypomastigotes (M), trypomastigotes deriving from cell cultures (T), amastigotes (A), and epimastigotes (E). The degree of significance between measurements was calculated using the Tukey-Kramer test, taking *P* values of <0.05 (*) as significant and *P* values of <0.001 (***) as extremely significant. The data represent the mean values and standard deviations for three independent experiments.

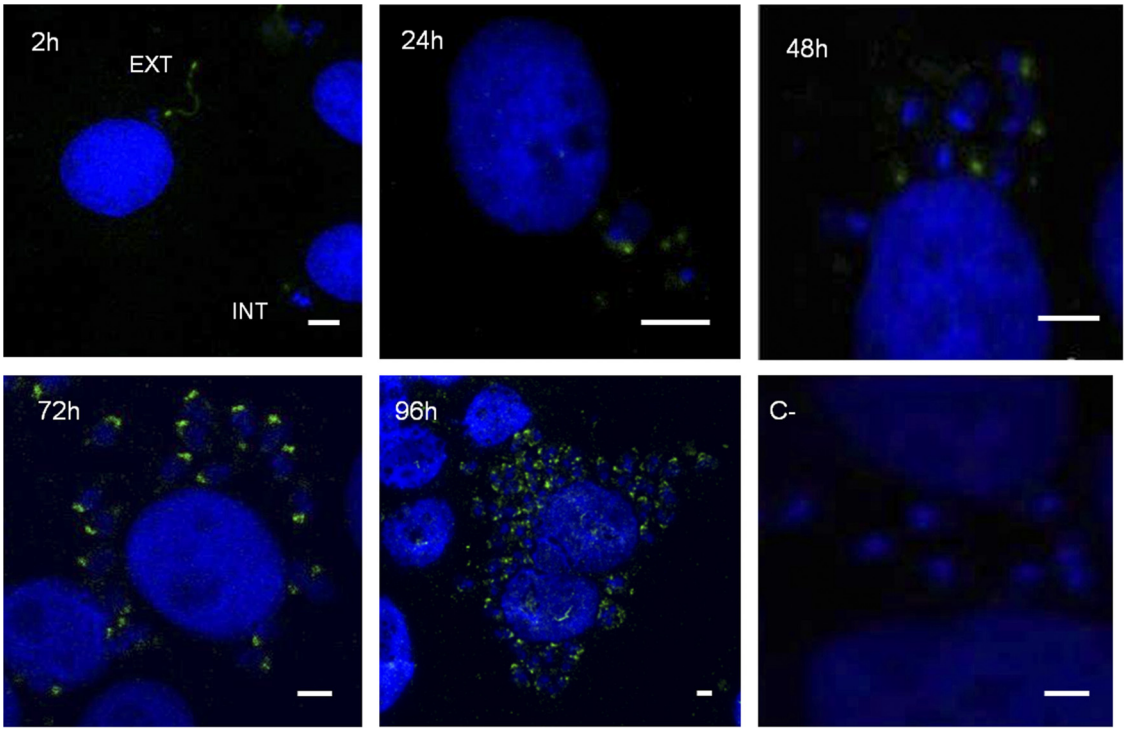

FIG 3 Immunofluorescence of the signal peptide of MASPs in the CL-Brener strain. Vero cells synchronized and infected with metacyclic trypomastigotes at a cell-to-parasite ratio of 1:5 were studied at 2, 24, 48, 72, and 96 h postinfection. The negative control was made with a serum from preimmune mice. Bar = 5 μm. We used DAPI as a counterstain for DNA labeling. EXT, exterior; INT, interior.

physiological alterations involved in changing from extracellular to intracellular forms, which would explain their higher expression during the intracellular trypomastigote and amastigote phases (4).

Among the most important characteristics of the MASP genes are their highly conserved 5′ and 3′ regions and a central hypervariable region (4, 12). As shown in Fig. 1, our experiments using these two conserved regions corroborate the idea that this multigene family does not share orthologous genes with other species of kinetoplastids, as has been reported before for *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* genomes (the tritryps) (13). It has been estimated that the main species of tritryps diverged between 200 and 500 million years ago (7, 13), from which time the multigene families characteristic of *T. cruzi* began to evolve, and thus the existence of the conserved regions of the MASP family constitute molecular markers for the *Trypanosoma cruzi* taxon.

Because the 5′ terminus of the members of the MASP family is highly conserved, we decided to use this region to measure (using qRT-PCR) the level of expression of this family in the different stages of the PAN4 and CL-Brener strains. As shown in Fig. 2, there is a clear dichotomy in the expression pattern of the MASP family in the four main *T. cruzi* forms of the PAN4 strain (belonging to the DTUI lineage) and the CL-Brener strain (belonging to the DTUVI lineage). The maximum levels of expression were found in the amastigote stage of the CL-Brener strain and in the trypomastigote stage from cell culture for the PAN4 and CL-Brener strains (Fig. 2). The high levels of expression of the T stage may be explained by the fact that this stage is that which survives most readily in adverse biochemical and physiological environments, for which it must be prepared in terms of its exposure to the immune system and its capacity to infect cells and to complete its life cycle in a new invertebrate host (11).

Recently, Tarleton et al. (17) studied the differences in genetic content between *T. cruzi* strains by comparative genome hybridization (CGH), using the CL-Brener strain as a template, and found a high-copy-number variation (CNV) between *T. cruzi* strains that affects mainly the highly populated regions of repeat regions, including the *T. cruzi* multigene families mentioned above. In addition, genome sequencing has been undertaken with a new *T. cruzi* strain, the Silvio X10/1 (DTUI) strain, which belongs to a phylogenetic group somewhat distant from that of the CL-Brener strain originally sequenced (DTUVI). This sequencing has revealed a contraction in the gene content of its multigene families, which theoretically increases the functional plasticity of the parasite between strains (1, 15).

Surprisingly, our results showed that the A form presented different expression patterns in the two strains assayed, which supports the idea that strains from different lineages vary genetically and thus may also vary biologically (8). These differences are also found in the TcSMUG L family of mucins, for which product expression and processing vary considerably between parasite strains (26). Given these results, we decided to study the behavior of the MASP family during the intracellular cycle of *T. cruzi*.

Antibodies against the SP (conserved N-terminal end) of the MASP family revealed an increase in MASP expression from 24 h postinfection onwards, with fluorescence intensity peaking (maximum expression) at 72 h, the time when the amastigote stage begins to differentiate from the trypomastigote stage (Fig. 3 and 4). After the parasite emerges from the parasitophore vacuole, it begins to differentiate into an amastigote (21), and the highest

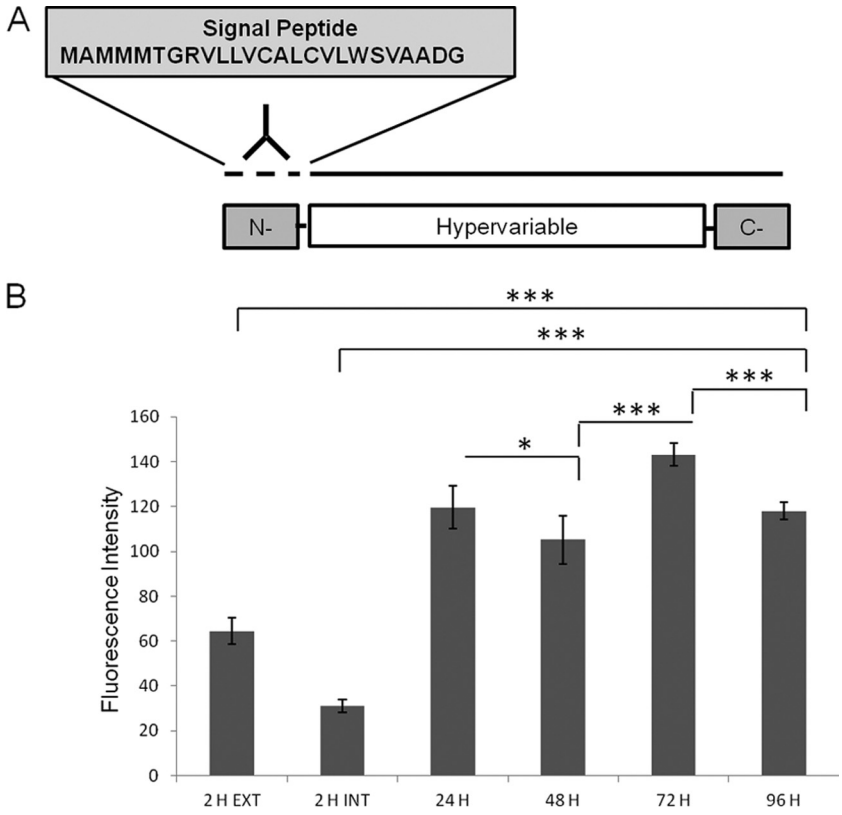

FIG 4 (A) Graphic representation of the location of the signal peptide of MASP as revealed by fluorescence. (B) Fluorescence intensities at 2, 24, 48, 72, and 96 h postinfection, obtained using an antibody against the signal peptide of MASPs. The intensities were measured over a constant area at the points where the green fluorescence of the parasites was located. The level of significance between the means was calculated by the Tukey-Kramer test, taking P values of <0.05 (*) as significant and P values of <0.001 (***) as extremely significant. The data represent the means ± standard deviations for measurements made with 100 amastigotes chosen at random. 2 H EXT, external metacyclic trypomastigotes; 2 H INT, internalized forms.

level of expression may be due in part to the amastigote's adaptation to the surrounding cytoplasm (6, 19, 22). In addition, this increase in MASP expression noted at 24 h postinfection may be related to the division processes of the amastigote, which occur between approximately 24 and 36 h after the invasion of the cell (10). The fact that MASP expression was higher in the A form than in the external M forms is in accordance with the results of the qRT-PCR assays. Nevertheless, as far as protein and mRNA levels are concerned, we found substantial differences between each A and M stage, indicating that part of the expressed RNA is not translated into protein. This might be attributed to the fact that some of this RNA could be transcribed as ncRNA and also that *masp* transcripts are found associated with TcDHH1, a DEAD box RNA helicase involved in mRNA metabolism, regulating the expression of at least epimastigote-specific genes (16).

The fact that not all parasites expressed these proteins between 24 and 48 h postinfection might indicate that expression of these proteins is an asynchronous process related either to asynchrony in parasite entry into the cell or to subsequent processes of multiplication after the initial division of the amastigote, although despite these differences in expression between some amastigotes and others, there was a clear increase in intensity in those that were expressing these proteins. These results support the hypothesis of the research group of Bartholomeu et al. (4), who proposed that the expression of MASPs may be clonal within a population of parasites, i.e., that these types of proteins are not always expressed in all parasites in a given sample.

The differences in expression between the forms of *Trypanosoma cruzi* during its intracellular cycle, together with the differences in expression between its various stages, and even strains, reflect the high regulation of this multigene family during the life cycle of the parasite.

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